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**GOVERNMENT OF INDIA  
MINISTRY OF COMMERCE & INDUSTRY,  
PATENT OFFICE, DELHI BRANCH,  
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NEW DELHI-110 008.**

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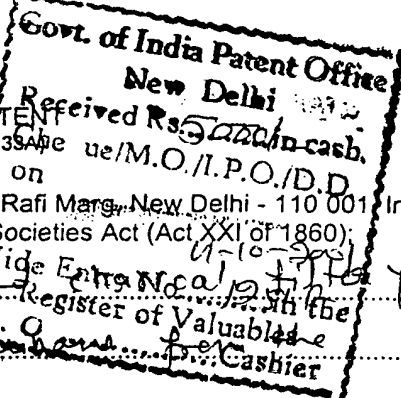
*I the undersigned being an officer duly authorized in  
accordance with the provision of the Patent Act, 1970  
hereby certify that annexed hereto is the true copy of the  
Application, Provisional Specification and Drawing sheets  
filed in connection with Application for Patent  
No.1025/Del/2001 dated 4<sup>th</sup> October 2001.*

*Witness my hand this 19<sup>th</sup> day of February 2002.*

**(H.C. BAKSHI)**  
Deputy Controller of Patents & Designs.



FORM 1  
THE PATENTS ACT, 1970  
(39 of 1970)  
APPLICATION FOR GRANT OF PATENT  
(See Sections 5(2), 7, 54 and 135 and rule 39A)



1. We, COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH, an Indian registered body incorporated under the Registration of Societies Act (Act XXI of 1860);  
2. hereby declare:  
(a) that we are in possession of an invention titled: *Act-N-ate*

*effectively reducing P-benzosem  
mainstream cigarette smoke.*

- (b) that the Provisional / ~~Complete~~ specification relating to this invention is filed with this application;  
(c) that there is no lawful ground of objection to the grant of patent to us;

3. further declare that the inventor(s) for the said invention is / are:

*Indu Bhushan Chatterjee, of DR. B.C. Guha  
Centre for genetic engineering and Biotechnology  
Calcutta University College of Science, 35, Ballygunge  
Circular Road, Kolkata 700019*

1025P 1  
04 OCT 2001

4. We, claim the priority from the application(s) filed in convention countries, particulars of which are as follows: NOT APPLICABLE  
5. We state that the said invention is an improvement in or modification of the invention, the particulars of which are as follows and of which we are the applicant:  
(a) Patent application no.:  
(b) Patent application date:  
6. We state that the application is divided out of our application, the particulars of which are given below and pray that this application deemed to have been filed on ..... under section 16 of the Act.  
(a) Patent application no.:  
(b) Date of filing provisional and / or complete specification ..... and .....  
7. That we are the assignee of the true and first inventor(s).  
8. That our address for service in India is as follows:

Head, IPM Division, CSIR,  
INSDOC Building, 14 Satsang Vihar Marg,  
New Delhi - 110 067.

Phone: 696 2560, 696 8819; Fax: 696 8819.

PTO

ORIGINAL

9. Following declaration was given by the inventor(s):

I / We the true and first inventor(s) for this invention declare that the applicants herein is / are my / our assignee:

Dated this <sup>4th</sup> 3rd day of October, 19/20 2001

Name (in full with expanded initials)

Signature of the true and first inventor(s)

INDU BHUSAN CHATTERJEE

*Indu Chatterjee*

10. That to the best of our knowledge, information and belief the fact and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to us on this application.

11. Followings are the attachment with the application:

(a) Provisional / ~~Complete~~ specification (3 copies).

(b) Drawings (3 copies).

(c) Priority document(s).

(d) Statement and Undertaking on FORM-3.

(e) Power of authority.

(f) Fee Rs. <sup>500</sup> in Cheque no.: 857659 dated: 12 Jun 2001

on State Bank of India, New Delhi Main Branch, Parliament Street, New Delhi - 110 001.

We request that a patent may be granted to us for the said invention.

Dated this <sup>4th</sup> 3rd day of October, 19/20 2001

*Indu Chatterjee*

SCIENTIST

Intellectual Property Management Division,  
Council of Scientific and Industrial Research.

To,  
The Controller of Patents,  
The Patent Office, New Delhi.

डा० (श्रीमती) इन्दु चटर्जी  
DR. (MRS.) INDU CHATTERJEE  
SCIENTIST  
आई.पी.एम.डी. (आर.एस.आई.आर.) I.P.M.D. (C.S.I.R.)  
14, सरसंग निहार मार्ग, सारसंग उपर मार्ग  
नई दिल्ली-110087/NEW DELHI-110087

**FORM 2**

**THE PATENTS ACT - 1970**

(39 of 1970)

**PROVISIONAL SPECIFICATION**

( See Section 10 )

**ACTIVATED CHARCOAL FILTER FOR EFFECTIVELY REDUCING P-  
BENZOSEMIQUINONE FROM THE MAINSTREAM CIGARETTE SMOKE**

1025 1  
04 OCT 2001

***COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH,***  
Rafi Marg, New Delhi-110001, India, an Indian Registered Body  
incorporated under the Registration of Societies Act (XXI of 1860).

ORIGINAL

The following specification particularly describes the nature of the invention and the manner in which it is to be performed :

**Title: Activated charcoal filter for effectively reducing p-benzosemiquinone from the mainstream cigarette smoke**

**Abstract**

This invention refers to cigarette smoke filters comprising stipulated amounts of specific grain sizes of activated charcoal for effectively reducing from the mainstream smoke the level of p-benzosemiquinone (p-BSQ), a relatively stable highly reactive major harmful oxidant, without significantly affecting the flavour and taste of the smoke while providing comfortable mouthful of smoke and nicotine delivery, so that the said charcoal filter cigarettes becomes potentially less hazardous safer cigarettes and may be acceptable to the smokers with marked reduction in health risk. P-BSQ is mainly responsible for the oxidative damage of protein and also DNA caused by cigarette smoke solution. P-BSQ in the smoke is not reduced by conventional cellulose acetate filters or commercial charcoal filters available in the market. The said charcoal filters also effectively reduce the level of nitric oxide and tar from the mainstream smoke.

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## Summary

The present invention describes cigarette smoke filters comprising stipulated amounts of specific grain sizes or mixture / combination of grain sizes of activated charcoal for effectively reducing p-benzosemiquinone (p-BSQ) from the mainstream smoke without significantly affecting the taste and flavour while providing comfortable mouthful of smoke and nicotine delivery. p-BSQ is a relatively stable free radical and a highly reactive major harmful oxidant present in the cigarette smoke, which is mainly responsible for the oxidative damage of protein as well as DNA. The different grain sizes or mixture of different grain sizes of activated charcoal have been selected from BS (British stand mesh) 25/44, 44/52, 52/60, 60/72 and 72/85. The level of p-BSQ in the smoke from different charcoal filters cigarettes is reduced to 55 – 85 percent, which is accompanied by inhibition of BSA oxidation to the extent of 55 – 82 percent. The charcoal filters also effectively reduce nitric oxide to 44 – 68 percent and tar to 10 – 50 percent from the mainstream smoke. Nicotine delivery, which is reduced to some extent by the charcoal filters, is replenished by fortification of the tobacco with nicotine without any increase in the p-BSQ level of the smoke, apparently because nicotine is not a precursor of p-BSQ.

## Background

Cigarette smoking is the world's single most preventable cause of disease and death. Worldwide, about 36 percent of all adults smoke cigarettes. According to a 1999 World Health Organization estimate, there are 4 million deaths a year from tobacco. Tobacco smoke contains more than 4000 compounds. Among these, nicotine is the habit forming pharmacological agent. Others are toxins, mutagens and carcinogens that cause or enhance various degenerative diseases including cancer of lung and other organs, chronic obstructive pulmonary disease such as bronchitis and emphysema as well as heart disease and stroke. Since approaches to cessation of smoking by public health campaigns and anti-smoking laws passed by local Governments have had limited success, the most practicable approach is the prevention of the hazardous effects caused by cigarette smoke. Modification of the cigarette is in itself a practical approach to reducing the toxic compounds contained in cigarette smoke. One of the approaches was to use cigarette filters. This is what the cigarette manufacturers have been trying to do for the last few decades. The cigarette companies have introduced cigarettes with filter tips to reduce the harmful compounds in the smoke, apparently to produce safer cigarettes without affecting the flavour and nicotine content of the smoke. There are four main types of filters in use to-day, namely, cellulose acetate, polypropylene, pure cellulose and filters containing granular additives, mainly activated charcoal. Cellulose acetate dominates the global filter market with 68 percent. Polypropylene filters follow with 21 percent (almost all of which are in China), charcoal filters comprise 10 percent and cellulose filters comprise less than 1 percent. Since it is difficult in selectively reducing specific compounds, the companies have focused on reducing the tar components, which is thought to contain the majority of harmful compounds. This was the reason of the wide utilization of cellulose acetate filter tips. While this process is effective for reducing some

portion of the tar, it is not selective for individual compounds, particularly the gaseous and vapour phase components of cigarette smoke. However, tar is a poor concept as a basis for regulating tobacco. It is known that different brands of cigarettes produce tars with greatly varying concentrations of key toxins. Many people smoke low tar / low nicotine products believing that smoking these products are safer or will reduce their risks of cancer and other diseases. However, in order to compensate for lower levels of nicotine, many smokers often take



bigger, deeper or more frequent puffs or smoke more cigarettes to obtain their needed levels of nicotine. As a result, their exposure to toxins may not be really reduced. This is why health scientists do not consider 'lights' or 'ultra lights' cigarettes as reliably less hazardous. In fact, till date there is no such thing as a safe cigarette. Obviously, such cigarettes with lower tar and nicotine content may be a distracting illusion of reduced harm and may not give any health benefit. This is particularly because the factors of cigarette smoke, which contribute to the known risks, are still not clearly defined. We consider that reducing the undesirable compounds in smoke is certainly of great importance, but selectively reducing the most undesirable compound is likely to be the most effective way of lowering the risk of smoking.

Activated charcoal filters seem to be better than cellulose acetate filters. These filters remove significant amounts of some toxic and irritant gases and semivolatile organic compounds, which the cellulose filters do not. However, there is presently no data directly linking the use of commercial charcoal filters to lowered cancer rates. It would have been ideal to pinpoint one compound or a group of compounds as the main culprit in cigarette smoke and to use a filter to selectively reduce this. Since the factors in cigarette smoke that contribute to the known risks are not clearly understood, a clear definition of a safer or lower risk cigarettes does not exist. In fact, there is no existing parameter by which toxicity or carcinogenic potential of a particular brand of cigarette can be measured.

Nevertheless, at present the most discussed carcinogens and toxins are the tobacco specific nitrosamines (TSNA) particularly, N-nitrosornicotine (NNN) and 4-(methylnitrosamino) -1-(3-pyridyl) -1-butanone (NNK), polynuclear aromatic hydrocarbons (PAH) such as benzo(a) pyrene, aldehydes (e.g. acetaldehyde, crotonaldehyde), volatile hydrocarbons (benzene, toluene), aromatic amines, trace materials as well as carbon monoxide, nitric oxide, acrolein and phenol. However, it is yet to be known which of these carcinogens toxins is most harmful and whether removal of all these will reduce the risks of smoking and incidence of cancer. For many years it has been believed that polycyclic aromatic hydrocarbons, particularly benzo(a) pyrene, play a major role in the development of lung cancer. Nowadays, TSNAs are the focus of a lot of attention. However, just because these compounds can cause cancer or other diseases on their own, they are not necessarily responsible for cancers or other diseases resulting from tobacco smoke. The carcinogens present in tobacco smoke are at such small concentrations that it is highly unlikely that one would cause cancer or other diseases on its own. For example, the

concentration of benzo(a) pyrene in the mainstream cigarette smoke is in the range of 10 to 40 ng (1) and the average amount of both NNK and NNN is 200 ng per cigarette (4). Moreover, not to date there has been any single compound identified as more responsible than others for the risks associated with smoking. As indicated before, it would have been ideal to pinpoint the most hazardous compound in cigarette smoke and to eliminate it by the use of filters.

We have reported before (3) that aqueous extract of cigarette smoke contains some stable oxidant, which causes extensive oxidative damage of proteins. Very recently, we have isolated the oxidant from cigarette smoke / tar solution and identified it as a major potentially hazardous compound, which almost quantitatively accounts for the oxidative damage of proteins caused by cigarette smoke solution. The molecular structure of the oxidant has been found to be p-benzosemiquinone (p-BSQ) as evidenced by elemental analysis, mass spectrum, uv, ir, nmr and esr spectra as well as by chemical properties. p-BSQ is a relatively stable free radical, apparently because the unpaired electron is delocalised over an aromatic framework containing heteroatoms leading to different mesomeric forms, namely, anionic, neutral and cationic forms. The half-life of p-BSQ, as determined by its oxidant activity, is 48 hours in the solid state at the room temperature and about 1.5 hours in aqueous solution at pH 7.4. We have examined 12 different brands of cigarettes including Indian, American, British, Russian and Japanese cigarettes. The content of p-BSQ in the mainstream smoke of these different brands varies from 104 µg to 200 µg depending on the brand of cigarette. Thus its concentration in the smoke is approximately 5000 to 11,000 times that of benzo(a) pyrene and 520 to 1000 times that of both NNK and NNN. Unlike PAH and TSNA, p-BSQ is a highly reactive strong oxidant which reacts directly with proteins. Besides being responsible for protein oxidation, pBSQ is also responsible for the oxidative damage of DNA. Since DNA oxidation is implicated with mutation and cancer, p-BSQ may be a major factor for the production of cancer caused by cigarette smoke.

free radicals are critically involved in causing DNA damage of a type that is not easily repaired and therefore may lead to mutation and cancer.

Earlier observations of Pryor and his associates (4) suggested that the principal relatively stable radical in cigarette tar might be quinone / hydroquinone / semiquinone complex which was an active redox system and that this redox system was capable of reducing molecular oxygen to produce superoxide, leading to hydrogen peroxide and hydroxyl radicals, which may eventually

lead to oxidative damage of biological macromolecules. Since cigarette tar was an incredibly complex mixture and since the tar radicals were not isolated and unambiguously identified, the conclusion of Pryor and his associates (4) concerning the chemistry or biochemistry of the tar radicals was regarded as tentative. The authors thought that the principal radical in tar was actually not a monoradical and probably not a single species. However, as mentioned before, we have observed that the major stable hazardous oxidant in cigarette smoke is a single species namely, p-BSQ. The oxidative damage of proteins produced by p-BSQ is not inhibited by SOD or catalase, affirming that the oxidative damage is not mediated by secondarily produced superoxide and hydrogen peroxide. We have further observed that p-BSQ oxidized protein in the nitrogen atmosphere in the absence of molecular oxygen (3), indicating that there is a direct interaction of p-BSQ and biological macromolecules.

The aforesaid results would indicate that p-BSQ, a major highly reactive harmful oxidant occurring in high concentrations in cigarette smoke, is possibly mainly responsible for the oxidative damage of proteins and DNA leading to degenerative diseases and cancer. It would thus appear that on the one hand p-BSQ content in the smoke might be a parameter of toxicity of a particular brand of cigarette and on the other hand elimination of p-BSQ from the mainstream smoke will produce potentially less hazardous safer cigarettes. We have observed that cellulose acetate filter is ineffective in absorbing p-BSQ, but activated charcoal filters adsorb it. Too much of charcoal in the filter not only eliminates p-BSQ but also drastically reduces the nicotine content and also alters the flavour and taste of the smoke. Contrariwise, too little charcoal is ineffective in significant reduction of p-BSQ. In fact, elimination of p-BSQ from the smoke depends on the amount of particular grain size or grain sizes of activated charcoal used. So, we have devised cigarette filters using stipulated amounts of specific grain sizes of charcoal to find out optimum filtering devices for effective reduction of p-BSQ. Since activated charcoal is known to adsorb significant amounts of many of the toxic gas and vapour phase components of cigarette smoke, the said activated charcoal filters are expected not only to remove p-BSQ, which is conceived to pose the greatest health risk, but many other toxic components thereby producing potentially less hazardous cigarettes.

Use of activated charcoal filters is not new. US patent No.4,038, 992 (1977), refers to a blend of granules of proteins and charcoal for removing deleterious compounds from tobacco smoke. US patent No.4,373,539 (1983) describes a charcoal filter of coiled plastic tube filled with activated

charcoal. US patent No.5,909,736 (1999) specifically refers to activated charcoal enriched with biological materials such as hemoglobin and lysates of erythrocytes. Activated charcoal has also been used in combination with perforated filters. This has contributed to drastic reduction in smoke yields of tar and nicotine. Such techniques are being used in some of the developed countries. Among the commercial charcoal filter cigarettes available in the market, about less than 1 percent of American cigarettes and 2 percent of Russian cigarettes use charcoal filters. However, charcoal is most popular in Japan. Out of the total Japanese cigarette market, about 95 percent have charcoal filters. Charcoal is also popular in South Korea, where the most widely used charcoal filters (about 90 percent) contain activated carbon blended with zeolite. In Hungary and Venezuela cigarette market, 90-95 percent have charcoal filters. In most cases, the charcoal filter contains small amount of activated charcoal granules distributed in some porous material or embedded within cellulose acetate filter. Charcoal filters in general reduce toxins in the smoke. But no evidence exists that the already available commercial charcoal filter cigarettes are significantly less dangerous for the users. We have examined one brand of Russian charcoal filter cigarette and one brand of mild Japanese charcoal filter cigarette containing low tar and low nicotine. The Russian cigarette had about 16 mg tar, 590  $\mu$ g nicotine and 128  $\mu$ g of p-BSQ in the mainstream smoke. The Japanese cigarette had about 12 mg tar, 500  $\mu$ g nicotine and 104  $\mu$ g p-BSQ in the smoke. The Russian cigarette contained about 10 mg of charcoal and the Japanese cigarette about 30 mg of charcoal scatteredly embedded in cellulose acetate fiber filter. We observed that the p-BSQ content of the smoke from both the cigarettes remained unaltered irrespective of whether the charcoal filter was present or replaced by similar length of conventional cellulose acetate filter. This would indicate that the charcoal filters incorporated in both the Russian and the Japanese cigarettes were ineffective in reducing p-BSQ content of the mainstream smoke. As would be expected. BSA oxidation by the aqueous extract of CS from

from the Russian cigarette ( $7.5 \pm 0.2$  nmoles of carbonyl / mg BSA) and mild Japanese cigarette ( $6.2 \pm 0.2$  nmoles of carbonyl / mg BSA) remained unaltered irrespective of whether the charcoal filters were present or replaced by similar length of conventional cellulose acetate filter.

## Object

The object of the present invention is to provide special activated charcoal filters mainly to reduce from the mainstream smoke p-benzosemiquinone (p-BSQ), a highly reactive major harmful oxidant, which is almost singly responsible for the oxidative damage of proteins and probably also DNA, thus conceived to pose the greatest health risk. The specific purpose is to use stipulated amounts of specific grain sizes or mixture of specific grain sizes of activated charcoal to produce potentially less hazardous cigarettes, without significantly affecting the taste and flavour while providing comfortable mouthful of smoke and nicotine delivery, so that the said charcoal filter cigarettes may be acceptable to the smokers with marked reduction in health risk. Although charcoal filters are commercially available, those are not effective in reducing the p-BSQ of the smoke. Nevertheless this invention may be considered to be a re-evaluation and improvement of the existing state of art. Since activated charcoal not only adsorbs p-BSQ but also some tar and nicotine, the said charcoal filter cigarettes may be categorized as relatively low tar, low nicotine mild cigarettes. Apprehending that there might be some smokers who would not like mild cigarettes with low nicotine delivery, the tobacco of some of the said charcoal filter cigarettes will be fortified with nicotine to produce regular cigarettes with comparable nicotine content without any increase in the p-BSQ level of the smoke.

## Methodology

### Construction of activated charcoal filter

The activated charcoal filter was constructed by placing stipulated amounts of different grain sizes or mixture of grain sizes of activated charcoal in a thin plastic tube, the inside diameter of which was same as the outside diameter of the tobacco portion of the cigarette or the conventional cellulose acetate filter. The plastic tube could be replaced by tubes manufactured of light grade materials, namely hard paper tube, plastic wrapped paper tube or tube made with aluminium foil. At the one end of the tube containing the charcoal was inserted the conventional cellulose acetate filter (approximately 10 - 14 mm) which constitutes the mouthpiece and at the other end was inserted the tobacco portion of the cigarette (approximately 63 mm). A thin section of cellulose acetate filter (approximately 3 mm) was placed in the cavity in between the tobacco portion and charcoal bed as depicted in the drawing (Fig. 1). Essentially, the charcoal filter is a cavity filter where the activated charcoal granules are placed in a void space between two segments of cellulose acetate filters. As mentioned above, one portion of the cellulose acetate filter ( $\approx 10 - 14$  mm) is the mouthpiece and other portion ( $\approx 3$  mm) constitutes a barrier between the charcoal bed and the tobacco portion (Fig. 1). The portions, namely, the cellulose acetate mouth piece, the charcoal filter, the thin cellulose acetate filter placed in between the charcoal and the tobacco portion and the tobacco portion all are constructed into one single unit (Fig. 1). The cellulose acetate filter does not necessarily improve the filtration of p-BSQ of the smoke. However, its use in cooperation with the charcoal filter adds to the convenience of using it as a mouthpiece for suction. The thin section of the cellulose acetate filter placed in between the charcoal and the tobacco portion was used to prevent any infiltration of charcoal granules into the tobacco of the cigarette. The length of the charcoal packed in the filter corresponded

approximately to the weight of charcoal used. For example, 100 mg charcoal corresponding to 5 mm, 200 mg charcoal, 20 mm and so on. The total length of a charcoal filter cigarette using 300 mg of charcoal is 91 mm [10 mm cellulose acetate filter as a mouthpiece, 15 mm charcoal bed, 3 mm cellulose acetate as a partition between charcoal bed and tobacco portion and 63 mm tobacco portion]. The length of the cellulose acetate may be varied, because it is practically ineffective in reducing p-BSQ of the smoke. The grain size of charcoal used has been expressed in the British Standard (BS) mesh. The size BS 25/44 means particles

passing through mesh 25 but retained on mesh 44. Similarly, BS 44/25 means particles passing through mesh 44 but retained on mesh 52. All other grain sizes used in this invention, namely BS 52/60, 60/72 and 72/85 are explained in the same way.

#### Measurement of p-benzosemiquinone (p-BSQ)

p-BSQ was quantitatively measured by HPLC as described before (Patent # 1). Five to ten micro liters of the filtered smoke solution was diluted with mobile solvent and 20  $\mu$ l of this diluted solution was injected to the HPLC column with the UV detector set at 294 nm. The parameters used were as follows.

Instrument	: Simadzu 10A
Column	: Silica column (Lichrospher <sup>®</sup> Si60, Merck)
Mobile solvent	: Methylene chloride : methanol (90 : 10, v/v)
Flow rate	: 0.5 ml / min
Pressure	: 29 Kgf / cm <sup>2</sup>
Retention time	: 8.808

The amount of p-BSQ present in the smoke solution was calculated from the peak area, taking 100 ng of p-BSQ corresponding to an arbitrary area of 1,90,000 obtained from a standard curve.

The efficacy of activated charcoal filters was also determined by measuring the comparative yields of p-BSQ. p-BSQ was isolated from cigarette smoke solution by fractional solvent extraction followed by band TLC as described before (Patent #1). After proper dilution of the TLC extract with the mobile solvent, 20 $\mu$ l of the diluted solution was injected to the HPLC column. p-BSQ was detected at 288 nm, the  $\lambda_{max}$  of p-BSQ in the mobile solvent used. The parameters used were as follows.

Instrument	: Simadzu 10A
Column	: Lichrospher <sup>®</sup> 100 RP-18 endcapped (5 $\mu$ m), Merck
Mobile solvent	: Water : methanol (95 : 5, v / v)
Flow rate	: 0.5 ml / min
Pressure	: 38 Kgf / cm <sup>2</sup>
Retention time	: 7.242 min

### **Measurement of oxidative damage of proteins**

Protein oxidation as evidenced by carbonyl formation was measured by reaction with 2,4-dinitrophenyl hydrazine similar to that done before in our laboratory (3). When BSA was used, the values were expressed as nmoles of carbonyl formed per mg BSA. The incubation system contained 1 mg BSA and 50  $\mu$ l of smoke solution obtained from cigarettes with or without charcoal filter in a final volume of 200  $\mu$ l of 50mM potassium phosphate buffer, pH 7.4. After incubation for 1 hr. at 37<sup>0</sup>C, the protein was precipitated with 200  $\mu$ l of trichloroacetic acid solution and the rest of the procedure followed as before (3). Oxidative damage of proteins was also measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of guinea lung microsomal proteins as described before (3).

### **Preparation of microsomes**

Guinea pig lung microsomes, washed free of ascorbic acid, were prepared as described before (3).

### **Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE of microsomal proteins was performed by the procedures similar to that described before (3).

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### Measurement of nicotine

Smoke from a lit cigarette was allowed to dissolve in 2 ml of 50mM potassium phosphate buffer, pH 7.4 and filtered through 0.45  $\mu$ m Millipore filter as described before (3). One milliliter of the yellow coloured filtrate was extracted with one milliliter of methylene chloride by vigorous vortexing to extract the nicotine in the methylene chloride layer. Five hundred microliter of the methylene chloride layer containing the nicotine was then vortexed with 500  $\mu$ l of 50 mM HCl solution and the nicotine of the HCl solution was estimated by HPLC analysis at 254 nm. Five to 10  $\mu$ l of the nicotine solution was diluted to 200  $\mu$ l with the mobile solvent and 20  $\mu$ l of this diluted solution was injected to the HPLC column. A standard solution of nicotine was prepared in a similar way and analyzed. The parameters used were:

Instrument	: Shimadzu 10A
Column	: Lichrospher <sup>®</sup> 100 RP-18 endcapped (5 $\mu$ m), Merck
Mobile solvent	: 50 mM KH <sub>2</sub> PO <sub>4</sub> solution : acetonitrile : methanol; (78 : 17 : 5, v/v) containing 1 mM sodium heptane sulfonate, pH 5.0
Flow rate	: 0.3 ml / min
Pressure	: 24 Kgf / cm <sup>2</sup>
Temperature	: 25 <sup>0</sup> C
Retention time	: 4.185 min.

The minimum amount of nicotine that could be detected by the HPLC analysis under the conditions was 10 ng.

### **Measurement of tar**

Tar was collected by placing a Millipore filter unit between the lit cigarette with or without charcoal filter and the tube connected to a vacuum pump (LKB, Sweden) using a suction of 30 cm water. The Millipore filter (0.22  $\mu\text{m}$ ) was changed every two minutes to avoid clogging of the filter. For each cigarette, 4 filters were used. After complete burning of the tobacco, the filters were dried in a vacuum desiccator and weighed. The difference in weight of the filters before and after collecting the particulate portion was the weight of the tar.

### **Measurement of nitric oxide in cigarette smoke solution**

Ten milliliter of air saturated 50 mM potassium phosphate buffer, pH 7.4 was taken in a 50 ml boiling tube with a side arm and a stopper with a hole. An Indian commercial cigarette was mounted in a tube that penetrated the hole in the stopper and dipped down in the buffer solution. The side arm was connected to a water pump. The cigarette was lit and the smoke from the whole cigarette was bubbled through the buffer solution by applying a suction of 4 cm water. A portion of the cigarette smoke solution thus produced was filtered through a 0.45  $\mu\text{m}$  Millipore filter and extracted thrice with equal volume of methylene chloride. The concentration of potassium nitrite in the aqueous layer was measured after proper dilution by diazotization using Griess reagent. A standard solution of  $\text{NaNO}_2$  was run side by side.

## Results

### Effects of charcoal filters on the p-BSQ, tar and nicotine contents of the mainstream smoke as well as inhibition of protein oxidation

Using charcoal filter comprising stipulated amounts of different grain sizes or mixture of grain sizes of activated charcoal, the p-BSQ contents of the mainstream smoke are markedly reduced (Table 1). We have indicated before (Patent # 1) that among all the compounds present in the smoke solution, only p-BSQ is singly responsible for protein oxidation. Fig. 2 shows that oxidation of BSA, as evidenced by carbonyl formation, is almost quantitatively correlated with the contents of p-BSQ present in the incubation medium. As would be expected, reduction of p-BSQ content in the smoke by the use of charcoal filter is accompanied by marked inhibition of BSA oxidation (Table 1). Use of charcoal filter also results in reduction of some tar and nicotine (Table 1). The most effective grain sizes of activated charcoal, expressed in British Standard (BS) mesh, are 44/52, 52/60, 60/72 and 72/85 used singly or in combination. Grain sizes larger than 44/52, namely 25/44 and 10/25 are not efficient even when used in comparatively large amounts. Use of large amounts of charcoal (0.4 g to 1.0 g) causes problem in suction of the smoke. Use of coconut shell activated charcoal did not have any added advantage over commercially available activated charcoal. The most effective charcoal filters, those markedly reduce p-BSQ content in the smoke without significantly affecting the suction and providing comfortable mouthful of smoke, as evidenced by a panel of middle aged smokers, are given in the Table 1. The charcoal filters comprise 0.2 and 0.3 g of BS 44/52, 0.2 and 0.3 g of BS 52/60, 0.15 and 0.2 g of BS 60/72, 0.1 and 0.15 g of BS 72/85, a mixture of 0.2 g of BS 44/52 and 0.1 g of BS 52/60, a mixture of 0.2 g of BS 44/52 and 0.1 g of BS 60/72, a mixture of 0.1 g of BS 44/52 and 0.1 g of BS 72/85, a mixture of 0.2g of BS 44/52 and 0.1 g of BS 72/85, a mixture of 0.15 g of BS 44/52 and 0.1 g of BS 72/85, a mixture of 0.1 g of BS 52/60 and 0.1 g of BS 60/72, a mixture of 0.1g of BS 52/60 and 0.1 g of 72/85, and a mixture of 0.1 g of BS 60/72 and 0.1 g of BS 72/85, a mixture of 0.1 g of BS 52/60 and 0.05 g of BS 72/85, and a mixture of 0.1 g of BS 60/72 and 0.05 g of BS 72/85.. With the said charcoal filters, reduction of p-BSQ was in the range of 55 to 85 percent, with a corresponding inhibition of BSA oxidation was in the range of 55 to 82 percent.

**Effect of fortification of tobacco of the charcoal filter cigarettes with nicotine on the p-BSQ, tar and nicotine delivery of the smoke from an Indian commercial cigarette**

Table 1 shows that the charcoal filter cigarettes mentioned in this invention are very effective for markedly reducing the content of p-BSQ, the major hazardous oxidant present in the mainstream smoke. Table 1 further shows that the tar and nicotine delivery of these charcoal filter cigarettes are also considerably reduced. These charcoal filter cigarettes may therefore be considered as potentially safer mild cigarette. Apprehending that there might be some committed smokers who would not like mild cigarette with low nicotine delivery, the tobacco of some of the charcoal filter cigarettes has been fortified with 2 mg nicotine per cigarette and the results are given in Table 2. The results indicate that fortification of tobacco with 2 mg nicotine per cigarette lead to increase the nicotine delivery of the smoke considerably. The increase in nicotine delivery is accompanied by increase in tar content (Table 2). Fortification of tobacco with 3 - 4 mg nicotine produces about 30 - 50 percent more nicotine delivery (results not shown). However, fortification of tobacco with nicotine does not lead to any increase of p-BSQ content of the smoke, apparently because nicotine is not a precursor of p-BSQ and it does not contribute to either the level of p-BSQ in smoke or oxidation of BSA by the smoke solution (Table 2). The results would indicate that although fortification of tobacco of the charcoal filter cigarettes results in increased nicotine delivery, but the said charcoal filter cigarettes remain potentially safer cigarettes.

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### **Effects of charcoal filters on the nitric oxide level in the smoke solution from an Indian commercial cigarette**

Nitric oxide (NO) is one of the most important free radicals in the gas phase of cigarette smoke. Some scientists think that NO may be implicated in the development of chronic obstructive pulmonary disease and emphysema in the smokers. Results presented in Table 3 indicates that activated charcoal filter is very effective in reducing the NO level in the mainstream smoke. Using a mixture 0.2 g of BS 44/52 and 0.1 g of BS 72/85, the percent inhibition in the NO is as high as 68.

### **Protective effect of charcoal filters on the cigarette smoke induced oxidative degradation of guinea pig lung microsomal proteins**

Fig.3 (lane 2) shows that cigarette smoke solution obtained from an Indian commercial cigarette causes extensive damage of guinea pig lung microsomal proteins as evidenced by SDS-PAGE. The figure further shows that the oxidative damage of microsomal proteins is markedly reduced when the said cigarette was equipped with activated charcoal filters, namely, BS 52/60, 0.3 g (lane 3); a mixture of BS 44/52, 0.2g and BS 72/85, 0.1g (lane 4); a mixture of BS 60/72, 0.1g and BS 72/85, 0.1g (lane 5).

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**Table-1. p-Benzosemiquinone (p-BSQ), BSA oxidation, nicotine delivery and tar contents in the smoke solution from an Indian commercial cigarette with stipulated amounts of different grain sizes of activated charcoal**

Sl.No.	Activated charcoal		P-BSQ content in smoke (µg)	Percent inhibition in p-BSQ content	BSA oxidation <sup>d</sup> (nmoles of carbonyl formed / mg BSA)	Percent inhibition in BSA oxidation	Nicotine Delivery (µg)	Tar content (mg)
	Grain size <sup>a</sup> (BS mesh) <sup>b</sup>	Weight (g)						
1	NIL	NIL	180	--	10.65	--	935	20
2	44/52 <sup>b</sup>	0.20	81	55	4.79	55	525	18
3	44/52	0.30	50	72	3.20	70	425	15
4	52/60	0.20	63	65	3.62	66	420	14
5	52/60	0.30	27	85	1.90	82	350	10
6	60/72	0.15	72	60	4.26	60	425	15
7	60/72	0.20	45	75	2.87	73	370	12
8	72/85	0.10	70	61	4.26	60	500	18
9	72/85	0.15	50	72	3.20	70	450	15
10	44/52 <sup>c</sup> + 52/60	0.20 + 0.10	49	73	3.20	70	400	14

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Sl.No.	Activated charcoal		P-BSQ content in smoke (µg)	Percent inhibition in p-BSQ content	BSA oxidation <sup>a</sup> (nmoles of carbonyl formed / mg BSA)	Percent inhibition in oxidation	Nicotine Delivery (µg)	Tar content (mg)
	Grain size (BS mesh) <sup>a</sup>	Weight (g)						
11	44/52 + 60/72	0.20 + 0.10	43	76	2.66	75	400	12
12	44/52 + 72/85	0.10 + 0.10	50	72	3.20	70	400	13
13	44/52 + 72/85	0.20 + 0.10	29	84	1.92	82	350	10
14	44/52 + 72/85	0.15 + 0.10	36	80	2.24	79	365	10
15	52/60 + 60/72	0.10 + 0.10	58	68	3.73	65	400	13
16	52/60 + 72/85	0.10 + 0.10	50	72	3.20	70	360	12
17	60/72 + 72/85	0.10 + 0.10	40	78	2.66	75	350	11
18	52/60 + 72/85	0.10 + 0.05	64	65	3.60	66	430	15
19	60/72 + 72/85	0.10 + 0.05	50	72	3.20	70	440	15

a, British standard b, BS 44/52 means particles passing through mesh 44, but retained on mesh 52. All other grain sizes mentioned in the Table are explained in the same way. c, Indicates mixture of the two grain sizes d, Amount of carbonyl formed by 50 µl of smoke solution. Details of the incubation system and measurement of carbonyl are given under Methodology Section.



**Table 2. Effect of fortification of tobacco of the charcoal filter cigarettes with nicotine on the p-BSQ, tar and nicotine delivery in the smoke solution of the smoke from an Indian commercial cigarette**

Sl.No.	Charcoal filter	Fortification with nicotine (mg) +	p-BSQ content ( $\mu$ g) *	Nicotine delivery* ( $\mu$ g) +	Tar (mg)	BSA oxidation nmoles of carbonyl formed per mg BSA *
1	None	None	180	935	20	10.60
2	BS 52/60, 0.3g	None	27	350	10	1.90
3	BS 52/60, 0.3g	2	27	610	14	1.95
4	BS 44/52, 0.2g + BS 52/60, 0.1g	None	79	400	14	3.20
5	BS 44/52, 0.2g + BS 52/60, 0.1g	2	79	670	17	3.25
6	BS 60/72, 0.2g	None	45	370	12	2.85
7	BS 60/72, 0.2g	2	45	650	16	2.80
8	BS 44/52, 0.2g + BS 60/72, 0.1g	None	43	400	12	2.66
9	BS 44/52, 0.2g + BS 60/72, 0.1g	2	43	650	16	2.65
10	BS 44/52, 0.1g + BS 72/85, 0.1g	None	50	400	13	3.20
11	BS 44/52, 0.1g + BS 72/85, 0.1g	2	50	700	17	3.25
12	BS 44/52, 0.2g + BS 72/85, 0.1g	None	29	350	10	1.90

Contd. Page 22

Sl.No.	Charcoal filter	Fortification with nicotine (mg) +	p-BSQ content (µg) *	Nicotine delivery* (µg) +	Tar (mg)	BSA oxidation nmoles of carbonyl from per mg BSA *
13	BS 44/52, 0.2g + BS 72/85, 0.1g	2	29	575	14	1.95
14	BS 44/52, 0.15g + BS 72/85, 0.1g	None	36	365	10	2.24
15	BS 44/52, 0.15g + BS 72/85, 0.1g	2	36	600	13	2.24
16	BS 52/60, 0.1g +BS 72/85, 0.1g	None	50	360	12	3.20
17	BS 52/60, 0.1g + BS 72/85, 0.1g	2	50	600	15	15
18	BS 60/72, 0.1g + BS 72/85, 0.1g	None	40	350	11	2.65
19	BS 60/72, 0.1g + BS 72/85, 0.1g	2	40	605	14	2.66

\* Values are means of four determinations; SD<10%

+ Fortification of the tobacco with 3 mg nicotine instead of 2 mg nicotine results in about 30 percent more delivery of nicotine in the smoke (results not shown)

**Table 3. Effects of charcoal filters on the nitric oxide level in the smoke solution from an Indian commercial cigarette**

Sl.No	Charcoal filter	Nitric oxide (µg)	% Inhibition in the NO level
1	None	62	--
2	BS 52/60, 0.3g	28	55
3	BS 60/72, 0.2g	35	44
4	BS 44/52, 0.15g + BS 72/85, 0.1g	21	66
5	BS 60/72, 0.1g + BS 72/85, 0.2g	34	45
6	BS 44/52, 0.2g + BS 60/72, 0.1g	30	52
7	BS 44/52, 0.2g + BS 72/85, 0.1g	20	68

*Dated 4<sup>th</sup> October 2001.*

*Rajesh Samra*

डॉ. (श्रीमती) सुजाता सामंतानी  
DR. (M.P.S.) SUJATA SAMTANI  
विज्ञानज्ञ/SCIENTIST

संस्था प्र.डी. (सी.एस.आई.एन.ए.)/I.P.M.D (C.S.I.R)  
10, चौक, विहार भाग/14, SATSANG VIHAR, WARE  
नं. (विहार) - 110029 NEW DELHI-110027

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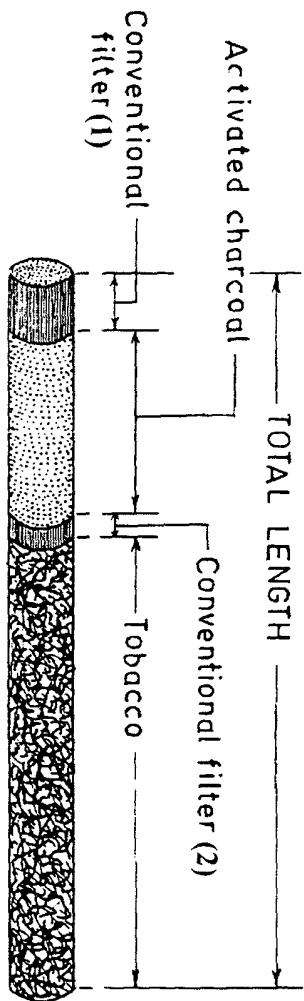
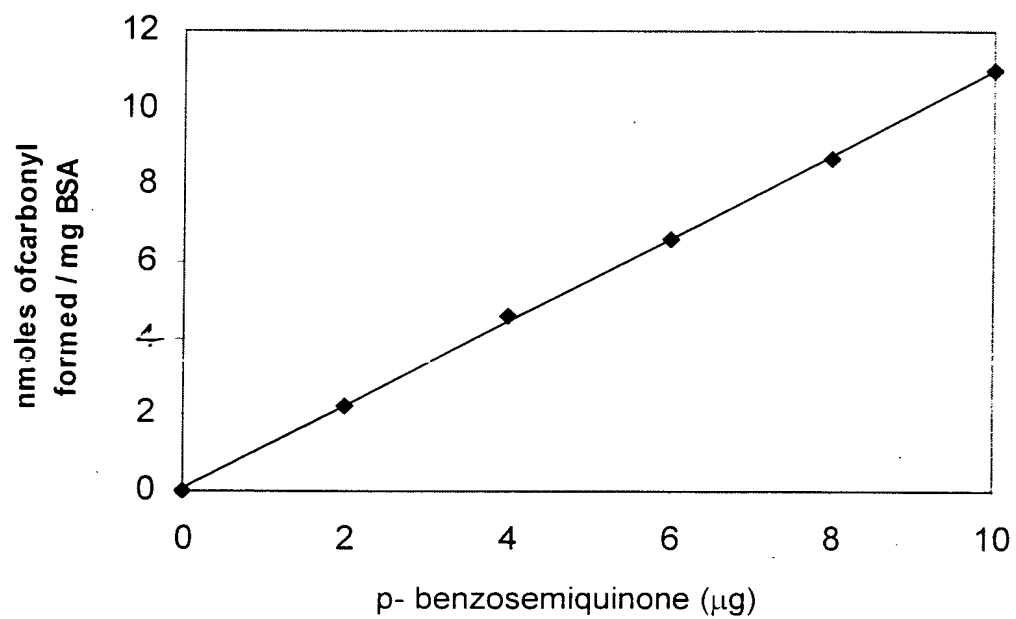


Fig 1. Drawing of a typical charcoal filter cigarette.

Conventional cellulose acetate fibre filter (1), acting as the mouthpiece, the length of which may vary according to convenience, e.g. 10-14 mm. Conventional cellulose acetate fibre filter (2), acting as a barrier between the charcoal bed and the tobacco portion to prevent infiltration of charcoal into tobacco, the length of which may be 2-4 mm. The length of the activated charcoal bed may vary depending on the amount of charcoal used, e.g. 4.5-5.5mm per 100 mg, 9-11 mm per 200 mg and 13-16 mm per 300 mg charcoal.

*Induvidy*  
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**Fig.2 Carbonyl formation in BSA by p-benzosemiquinone**

Indira Devi  
APPLICANTS





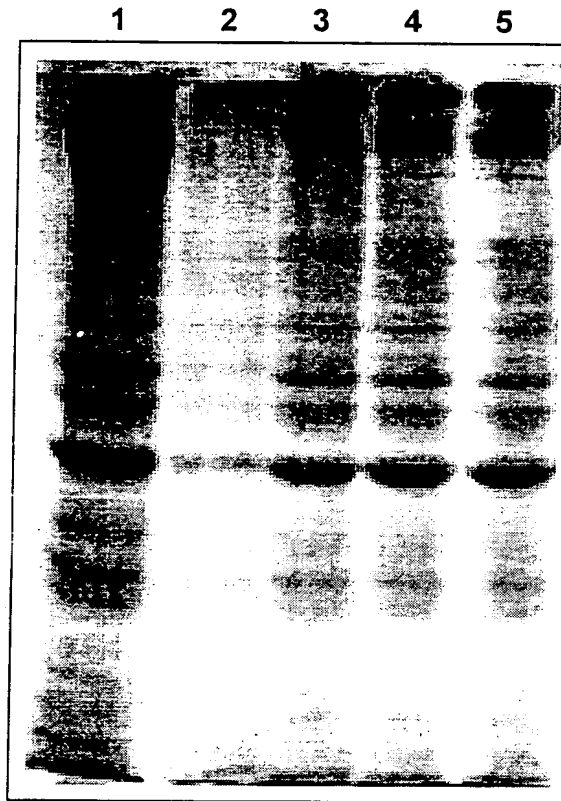


Fig.3. SDS-PAGE showing protective effect of charcoal filters on the cigarette smoke induced oxidative degradation of guinea pig lung microsomal proteins.

Lane1, microsomes incubated in the absence of cigarette smoke solution; lane2, microsomes incubated in the presence of solution of smoke from cigarettes without any charcoal filter; lanes 3-5, microsomes incubated with smoke solution from cigarettes having charcoal filters; lane 3, BS 52/60, 0.3g; lane 4, a mixture of BS 44/52, 0.2g and BS 72/85, 0.1g; lane 5, a mixture of BS 60/72, 0.1g and BS 72/85, 0.1g. In each case, the microsomal suspension( 1mg protein) was incubated with 50 $\mu$ l smoke solution in a final volume of 200  $\mu$ l of 50 mM potassium phosphate buffer, pH 7.4 for 2 hours at 37°C. After incubation, 40  $\mu$ l of the incubation mixture was subjected to 10% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R-250

*Indira Sundar*  
 APPLICANTS

